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**Expression of secretory leukocyte protease inhibitor and elafin in human fallopian tube
and in an in-vitro model of *Chlamydia trachomatis* infection**

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Key words: Secretory leukocyte protease inhibitor elafin *Chlamydia trachomatis*, ectopic pregnancy, Fallopian tube

Abstract

BACKGROUND Secretory leukocyte protease inhibitor (SLPI) and elafin are anti-protease and anti-microbial molecules with a role in innate immune defence. They have been demonstrated at multiple mucosal surfaces including those of the female reproductive tract.

METHODS AND RESULTS This study details their expression in human Fallopian tubes (ampullary region) throughout the menstrual cycle ($n = 18$) and from women with ectopic pregnancy ($n = 6$), and examined their regulation by infection with *Chlamydia trachomatis* in an in-vitro model. Quantitative real-time PCR analysis showed that SLPI and elafin were constitutively expressed in the Fallopian tube during the menstrual cycle but were increased in ectopic pregnancy ($P < 0.05$ versus early-mid luteal phase, $P < 0.01$ versus all phases, respectively). SLPI and elafin were immunolocalised to the Fallopian tube epithelium in biopsies from non-pregnant women and those with ectopic pregnancy. An in-vitro culture model of *C. trachomatis* infection of the OE-E6/E7 oviductal epithelial cell line showed that elafin mRNA expression was upregulated in response to chlamydial infection.

CONCLUSION These data suggest that SLPI and elafin have a role in the innate immune defence of the Fallopian tube in infection and ectopic pregnancy. Their role is likely to include regulation of protease activity, wound healing and tissue remodelling.

Introduction

The female reproductive tract must allow fertilisation, implantation and pregnancy to occur while maintaining the ability to respond to infection. Genital tract infection is associated with infertility, ectopic pregnancy and preterm labour and the innate immune response is an important component of the mucosal defence system (Horne *et al.*, 2008).

Natural antimicrobial molecules are effectors of the innate immune system and are widely expressed throughout the female reproductive tract. Secretory leukocyte protease inhibitor (SLPI) and elafin are anti-protease molecules, which also have antimicrobial activity (Hiemstra *et al.*, 1996, Sallenave, 2002, Simpson *et al.*, 1999, Simpson *et al.*, 2001, Tomee *et al.*, 1998, Wiedow *et al.*, 1998). SLPI inhibits several serine proteases including neutrophil elastase and cathepsin G (Thompson and Ohlsson, 1986) while elafin shows a more restricted activity, inhibiting only neutrophil elastase and proteinase 3 (Sallenave and Ryle, 1991, Wiedow *et al.*, 1991, Wiedow *et al.*, 1990). In addition to their anti-protease and antimicrobial roles, SLPI and elafin are reported to have a range of other anti-inflammatory properties, such as an ability to inhibit the nuclear factor-kappa B signalling pathway and modulation of the response to lipopolysaccharide (LPS) (Jin *et al.*, 1997, Sallenave *et al.*, 2003). We have previously reported that SLPI and elafin are expressed in human endometrium where they are regulated in a cycle dependent manner and our previous studies have also shown that SLPI is a progesterone regulated gene (King *et al.*, 2000, King *et al.*, 2003b, King *et al.*, 2003c). SLPI is expressed in endometrium from the mid-late secretory phase of the menstrual cycle when it is localised predominantly to the glandular epithelium (King *et al.*, 2000). Expression is maintained in early pregnancy although localisation shifts to the decidualised stroma. In contrast, elafin is expressed primarily in endometrial neutrophils during menstruation (King *et al.*, 2003b). Both molecules have also been reported to be present in cervix and vagina (Fichorova and Anderson, 1999, Moriyama *et al.*, 1999, Narvekar *et al.*, 2007, Pfundt *et al.*, 1996, Valore *et al.*, 2006) and in addition, SLPI has been shown to be present in human Fallopian tube where it is suggested to have a role in the protection of sperm from elastase (Ota *et al.*, 2002a). There are no studies documenting the expression of SLPI and elafin in Fallopian tube from different menstrual cycle phases.

The Fallopian tube is the most common site of ectopic pregnancy with over 98% of such pregnancies occurring in the oviduct (Lemus, 2000). Early pregnancy bleeding as a result of ruptured tubal pregnancy remains one of the commonest causes of maternal death during the first trimester of pregnancy (Farquhar, 2005). The pathology underlying ectopic pregnancy is

unclear although previous infection with *Chlamydia trachomatis* is a risk factor (Faro, 1991, Farquhar, 2005). The role of the innate immune system in chlamydial infection and ectopic pregnancy is not well understood. We have previously documented increased SLPI mRNA expression in the decidualised endometrium of women with ectopic pregnancy (Dalgetty *et al.*, 2008). However, there are no studies examining localisation and expression of SLPI and elafin in the Fallopian tube during ectopic pregnancy.

The aim of the current study was to examine the localisation and regulation of SLPI and elafin in the Fallopian tube from different menstrual cycle phases and in ectopic pregnancy and to examine regulation of SLPI and elafin during *in vitro* chlamydial infection of an oviductal cell line.

Methods

Tissue collection

Ethical approval for this study was obtained from Lothian Research Ethics Committee (04/S1103/20). All women were aged 18–45 years (mean 38). Written and informed consent was obtained from all patients before sample collection. Fallopian tube biopsies (all from ampullary region), endometrial biopsies (for histological dating) and sera (for measurement of circulating estradiol and progesterone concentrations for endocrine staging) were collected from women with regular menstrual cycles (21–35 days) undergoing gynecological procedures for benign conditions who had no previous history of ectopic pregnancy and had not taken any hormonal preparations in the three months prior to surgery ($n = 18$; mean age 39 years; see Table I). Fallopian tube was also obtained from women undergoing surgical management of tubal ectopic pregnancy ($n = 6$; mean age 37 years, mean gestation 58.09 days \pm SD 8.28, mean serum progesterone 58.53 nmol/L \pm SD 47.22). None of the women undergoing surgical management of ectopic pregnancy presented acutely with haemodynamic shock, and all required serial serum beta-HCG and ultrasound monitoring prior to diagnosis. Part of the Fallopian tube was immersed in RNAlater™ (Ambion, Texas, USA) at 4°C overnight then flash frozen at -70°C , and part of the Fallopian tube and the endometrial biopsies were fixed in 10% neutral buffered formalin overnight at 4°C, stored in 70% ethanol, and wax embedded. The endometrial biopsies underwent haematoxylin and eosin staining and dating by an expert histopathologist.

Quantitative RT-PCR

RNA was extracted from cells/tissues as detailed in the manufacturer's protocol (Qiagen, RNeasy mini kits). All samples were treated with DNase I (Qiagen) in order to remove any contaminating genomic DNA. Quantitative real-time RT-PCR was used to measure mRNA levels of SLPI and elafin. Complementary DNA (cDNA) was prepared in 20 µl reaction volumes containing: 1xRT buffer, magnesium chloride, dNTPs, random hexamers, RNase inhibitor and Multiscribe reverse transcriptase (Applied Biosystems, Cheshire, UK). 200 ng of template RNA were reverse transcribed and each cDNA experiment included two controls: one containing template RNA but no reverse transcriptase (RT negative) and the other containing reverse transcriptase with water in place of template RNA (RT H₂O).

PCR reaction mixtures contained Taqman 2x Master-mix (1x; Applied Biosystems), forward and reverse primers (300 nM; Eurogentec) and probe (200 nM; Eurogentec) for SLPI or elafin and forward and reverse primers and probe for ribosomal 18S (all 50 nM; Applied Biosystems). Ribosomal 18S was used as a housekeeping gene. Internal control (SLPI, liver; elafin, menstrual endometrium) and negative control (water in place of cDNA) samples were included in each PCR run along with the RT negative and RT H₂O control samples described above. All samples were analysed in triplicate using the $2^{-\Delta\Delta C_t}$ method. PCR reactions were run on an ABI 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, USA). SLPI and elafin primers and probes were designed using Primer Express software and their sequences have been reported previously (Dalgetty *et al.*, 2008).

Immunohistochemistry

Immunohistochemical localisation of SLPI and elafin was performed on Fallopian tube sections using standard protocols. In brief, tissue sections were dewaxed in xylene and rehydrated in descending grades of alcohol. Sections were microwaved for 15 minutes in antigen unmasking solution (Vector) and then non-specific endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Sigma-Aldrich). Sections then underwent avidin, biotin (Avidin biotin blocking kit, Vector) and protein blocks (DakoCytomation protein block, Dako), each for 10 minutes at room temperature. Sections were incubated overnight at 4°C with either mouse-anti SLPI (1:100; Hycult) or rabbit anti-elafin (1:600, kind gift from Jean-Michel Sallenave) (Sallenave *et al.*, 1994) antibody diluted in Dako REAL antibody diluent (Dako). In negative control sections, the primary antibody was substituted with mouse immunoglobulin (Ig)G (SLPI) or antibody diluent (elafin). Sections were subsequently incubated with biotinylated horse anti-mouse or goat anti-rabbit Ig and were then subjected to

an avidin biotin peroxidase detection system (both for 30 minutes at room temperature; Vectastain Elite ABC, Vector). Positive staining was detected using the peroxidase substrate, diaminobenzidine (ImmPACT DAB; Vector). Sections were counterstained with Harris' haematoxylin, dehydrated in ascending grades of ethanol and mounted from xylene in Pertex.

***C. trachomatis* detection in fallopian tube biopsies by PCR**

All Fallopian tube biopsies included in this study were screened for current chlamydial infection by PCR. DNA was extracted from whole Fallopian tube biopsies from the ampullary region of the tube, as detailed in the manufacturers' protocol (Qiagen, West Sussex, UK). The PCR protocol used a well-validated, in-house, plasmid-based methodology (kindly developed and designed by the West of Scotland Specialist Virology Centre).

Cell culture

The OE-E6/E7 oviductal epithelial cell line (a gift from the University of Hong Kong) (Lee *et al.*, 2001) was used in an *in vitro* model of chlamydial infection. Cells were maintained in Dulbecco's modified Eagle's medium F12 (Invitrogen) containing 10% fetal calf serum. Cells were plated in 48 well culture plates at a density of 2×10^5 cells/well. Prior to treatment cells were serum starved overnight and then washed with phosphate-buffered saline. Treatments were then applied in a 500 µl volume and performed in triplicate. Cells were exposed to live *C. trachomatis* at multiplicities of infection of 0.001, 0.01 and 0.1 for 4, 24 and 48 hours. To control for the presence of modulatory factors in the *C. trachomatis* stock and in the oviductal cell lysate resulting from infection, cells were also exposed to UVC-irradiated *C. trachomatis* stock and cell lysate, respectively. Uninfected cells and cells treated with LPS from *Salmonella minnesota* were included as additional controls. Cells were collected at each time point for subsequent RNA extraction and analysis of SLPI and elafin mRNA expression by real-time quantitative RT-PCR (as detailed above).

Statistical analysis

Data were logarithmically transformed prior to statistical analysis. Significant difference was determined by one-way analysis of variance and Tukey's posthoc analysis. A $P < 0.05$ was considered statistically significant.

Results

Expression of SLPI and elafin mRNA in fallopian tube during the menstrual cycle and in ectopic pregnancy

SLPI mRNA was expressed in Fallopian tube biopsies from throughout the menstrual cycle and did not vary at different cycle phases. The presence of ectopic pregnancy significantly increased expression of SLPI mRNA compared to the early-mid luteal phase of the menstrual cycle (Fig. 1a: $P < 0.05$).

Elafin mRNA expression was very low in Fallopian tubes from non-pregnant women and expression did not vary at different cycle phases. Ectopic pregnancy resulted in increased elafin mRNA expression in the Fallopian tube (Fig. 1b: $P < 0.01$).

Immunohistochemical localisation of SLPI and elafin in fallopian tube during the menstrual cycle and in ectopic pregnancy

SLPI was localised to the epithelium of all Fallopian tubes collected during the follicular phase of the menstrual cycle (all five cases) (Fig. 2a). Epithelial staining was also present in some Fallopian tubes (two out of five cases) from the early to mid-luteal (Fig. 2b) and late luteal-menstrual phases (two out of three cases). In the Fallopian tube from women with ectopic pregnancies, the localisation was similarly epithelial (Fig. 2c).

Elafin was also localised to the epithelium of Fallopian tubes from all cycle phases (Fig. 2d & e). In addition, in some Fallopian tube biopsies from ectopic pregnancy elafin was localised to leukocytes present in blood vessels within the mucosal layer (Fig. 2f).

Detection of current chlamydial infection in fallopian tube biopsies

All Fallopian tube biopsies were screened for current *C. trachomatis* infection by PCR and shown to be negative (data not shown).

Regulation of SLPI and elafin MRNA expression in an in vitro model of c.trachomatis infection

Infection of the OE-E6/E7 oviductal epithelial cell line with *C. trachomatis* had no effect on SLPI mRNA expression at 4 (data not shown), 24 and 48 hr time points (Fig. 3a). In contrast, elafin mRNA expression was increased by the highest dose of *C. trachomatis* at 24 hours

($P < 0.05$; Fig. 3b). There were no significant changes in elafin mRNA expression at 4 (data not shown) or 48 hours (Fig. 3b).

Discussion

In this study we have shown that the anti-protease and anti-microbial molecules, SLPI and elafin, are expressed in the epithelial layer of the human Fallopian tube throughout the menstrual cycle. Both molecules are present at other mucosal layers, such as the lung (Franken *et al.*, 1989, Sallenave *et al.*, 1992) and they are also widely expressed at reproductive sites including the vagina, cervix and endometrium, where they contribute to the innate immune response (Horne *et al.*, 2008). The presence of SLPI and elafin in the Fallopian tube suggests that they also have an innate immune role at this upper genital tract site, where they may protect against ascending infection and regulate associated inflammatory events. A previous study has suggested that the anti-protease actions of SLPI in the Fallopian tube may protect sperm from the detrimental effects of elastase (Ota *et al.*, 2002b) suggesting that SLPI and elafin may also modulate inflammatory events occurring at fertilisation which may be detrimental to sperm and the developing embryo.

We have shown that SLPI and elafin are not regulated in a cycle dependent manner at the mRNA level in the Fallopian tube. This is in contrast to our previous studies in endometrium, which have shown SLPI to be maximally expressed in the secretory phase of the menstrual cycle (King *et al.*, 2000) and elafin expression to peak at menstruation (King *et al.*, 2003b). These differences in regulation of the molecules at two upper genital tract sites may reflect the different functions of the Fallopian tube and endometrium. The endometrium undergoes repeat cycles of proliferation, differentiation and repair in response to changes in the levels of circulating steroid hormones (Jabbour *et al.*, 2006). It must allow the breaching of the mucosal barrier at implantation and menstruation while maintaining the ability to mount an innate immune response if necessary. Increased natural antimicrobial expression during the implantation window and at menstruation may be one mechanism for enhancing innate immune protection at these vulnerable times (King *et al.*, 2003a). In contrast, the Fallopian tube undergoes relatively few cycle dependent changes and the epithelial layer is not normally breached, and the constant expression of SLPI and elafin may reflect this.

Ectopic implantation occurs most commonly in the Fallopian tube (Lemus, 2000) although few studies have examined the molecular changes that predispose to or result from inappropriate implantation. In the current study we document increased mRNA expression of both SLPI and elafin in the Fallopian tube from ectopic pregnancies. Due to ethical

constraints it is not possible to obtain Fallopian tubes from women with intrauterine pregnancies and thus, it is not clear whether the increased expression of SLPI and elafin are a result of the systemic hormonal environment created by pregnancy or the local inflammatory changes that accompany ectopic pregnancy or prior tubal infection. Our previous studies have shown that SLPI is a progesterone responsive gene (King *et al.*, 2003c) and the increased mRNA expression present in the Fallopian tube in ectopic pregnancy may reflect high circulating progesterone levels. While histological studies suggest there is little evidence for acute inflammatory changes at the site of an ectopic pregnancy (Kutluay *et al.*, 1994), recent studies at the molecular level suggest that there are inflammatory changes. Disruption of the interleukin (IL)-1 signalling system is reported to occur in Fallopian tube with ectopic pregnancy (Huang *et al.*, 2005) and expression of leukaemia inhibitory factor, a cytokine known to have a crucial role in uterine implantation, is reported to be increased in tubal pregnancy (Ji *et al.*, 2008). Elafin and, to a lesser extent, SLPI are upregulated by inflammatory stimuli, including IL-1 (King *et al.*, 2003c, Sallenave *et al.*, 1994, Williams *et al.*, 2006), and a shift towards a pro-inflammatory environment may result in their increased expression in ectopic pregnancy. Previous infection with *C. trachomatis* is a risk factor for ectopic pregnancy and we have shown that elafin is upregulated in an oviduct epithelial cell line in response to chlamydial infection *in vitro*. These data suggest that *C. trachomatis* infection may contribute to the raised mRNA expression of elafin in ectopic pregnancy. The women recruited to this study were not currently infected by *C. trachomatis* but it is possible that they may previously have been infected with *Chlamydia*. This would require further serological analysis for IgG antibodies or chlamydial heat-shock protein 60 and may be important, as the link between *C. trachomatis* and ectopic pregnancy is mainly based on early seroepidemiological case-control studies (Bakken, 2008).

However, if *C. trachomatis* and ectopic pregnancy are linked, the role of SLPI and elafin in *C. trachomatis* infection and/or ectopic pregnancy is likely to include inhibition of protease activity (Thompson and Ohlsson, 1986, Sallenave and Ryle, 1991, Wiedow *et al.*, 1991, Wiedow *et al.*, 1990), promotion of wound healing (Ashcroft *et al.*, 2000) and tissue remodelling (Lee *et al.*, 2002). These anti-inflammatory actions will limit tissue damage due to infection or abnormal implantation. Alternatively, abnormal expression of SLPI and elafin may predispose to infection or ectopic pregnancy. Previous studies have suggested that changes in the innate immune system are associated with reproductive tract pathologies. Women with single nucleotide polymorphisms in two or more of the genes for Toll-like

receptor (TLR)9, TLR4, CD14 or nucleotide-binding oligomerization domain containing 2 (NOD2) showed a trend towards increased likelihood of tubal pathology (den Hartog *et al.*, 2006). Decreased expression of SLPI in vaginal secretions is related to infection of the lower genital tract by *C. trachomatis*, although it is unclear whether this occurs prior to or as a result of infection (Draper *et al.*, 2000). Contrary to our findings with OE-E6/E7 cells, experimental infection of HeLa cells with *C. trachomatis* results in increased expression of SLPI mRNA and SLPI peptide (Wheelhouse *et al.*, 2008). Despite this increase in SLPI expression following infection and the fact that HeLa cells constitutively produce SLPI in the absence of infection, *C. trachomatis* grows well, strongly suggesting that SLPI does not have a direct antimicrobial effect on chlamydial growth.

In summary, we have demonstrated that the anti-protease and anti-microbial molecules SLPI and elafin are expressed in human Fallopian tube, that mRNAs for both are upregulated in ectopic pregnancy, and mRNA for elafin is increased in response to chlamydial infection in a cell culture model. This suggests that SLPI and elafin are involved in the innate immune protection of the Fallopian tube during the normal menstrual cycle and may be important in the event of pathological conditions, such as infection and ectopic implantation.

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Legends

Table I

Demographics for Fallopian tube and endometrial biopsies from women undergoing surgery for benign gynecological conditions

Figure 1

Secretory leukocyte protease inhibitor (SLPI) and elafin mRNA expression in the human Fallopian tube (ampullary region) during the menstrual cycle and in ectopic pregnancy. F=follicular phase, EL/ML = early-mid luteal phase, LL/M=late luteal-menstrual phase. (a) SLPI mRNA expression. a = $P < 0.05$ (EL/ML versus ectopic). (b) Elafin mRNA expression. ab: $P < 0.001$ (F and EL/ML versus ectopic), c: $P < 0.01$ (LL/M versus ectopic). Data are mean \pm SEM.

Figure 2

Immunohistochemical localisation of SLPI and elafin in human Fallopian tube (ampullary region) during the menstrual cycle and in ectopic pregnancy. (a) SLPI. Follicular phase. (b) SLPI. Luteal phase. (c) SLPI. Ectopic. (d) Elafin. Follicular phase. (e) Elafin. Luteal phase. (f) Elafin. Ectopic. Negative controls in insets. Scale bar = 100 μ m.

Figure 3

SLPI and elafin mRNA expression in the OE-E6/E7 oviductal epithelial cell line upon exposure to *Chlamydia trachomatis*. MOI = multiplicity of infection, UV-C = ultra-violet irradiated *C. trachomatis* (control), Smin LPS = *Salmonella minnesota* lipopolysaccharide (control) (a) SLPI mRNA expression. (b) Elafin mRNA expression. abc: $P < 0.05$ (infection at 24 hours with 0.1 MOI versus media, lysate and Smin LPS controls). Data are mean \pm SEM.

Tab.I

Age (years)	Parity	Cycle phase	Serum estradiol (pmol/L)	Serum Progesterone (nmol/L)	Endometrium	Surgery	Reason for surgery	Uterine pathology
41	2	Follicular	1022.87	0.81	Proliferative	TAH	HMB	Adenomyosis
37	2	Follicular	940.44	3.82	Proliferative	TAH	HMB, pelvic pain	Adenomyosis
44	3	Follicular	829.42	4.19	Proliferative	TAH	HMB, pelvic pain	Adenomyosis, fibroid
36	3	Follicular	770.63	5.16	Proliferative	TAH	HMB	No abnormality
44	2	Follicular	116.3	2.88	Proliferative	STAH	HMB, dysmenorrhoea	No abnormality
40	5	Early luteal	255	49.05	Early secretory	TAH	HMB, dysmenorrhoea	No abnormality
43	2	Early-mid luteal	399.8	80.45	Early secretory	TAH	HMB, dysmenorrhoea	Adenomyosis, polyp, fibroid
34	2	Mid luteal	471	62.65	Mid secretory	LAVH	HMB, dysmenorrhoea	Adenomyosis, polyp, fibroid
40	2	Mid luteal	242	53.1	Mid secretory	TAH	HMB, dysmenorrhoea	No abnormality
35	2	Mid luteal	424	76.9	Mid secretory	TAH	pelvic pain	No abnormality
38	3	Mid luteal	266	37.1	Mid secretory	TAH	HMB	No abnormality
32	1	Mid luteal	549.91	88	Mid secretory	TAH	Dysmenorrhoea	No abnormality
43	2	Mid luteal	201	24.55	Mid secretory	TAH	HMB, pelvic pain	Fibroid
40	1	Mid luteal	1633.0	54.38	Mid secretory	TAH	HMB, dysmenorrhoea	No abnormality
37	2	Late luteal	392.18	3.06	Late secretory	TAH	Dysmenorrhoea	No abnormality
43	2	Late luteal	274	35.8	Late secretory	TAH	HMB	Fibroids
35	4	Menstrual	73	2.67	Menstrual	TAH	HMB, dysmenorrhoea	Adenomyosis
42	2	Menstrual	55	15.05	Menstrual	TAH	HMB	No abnormality

Fig.1

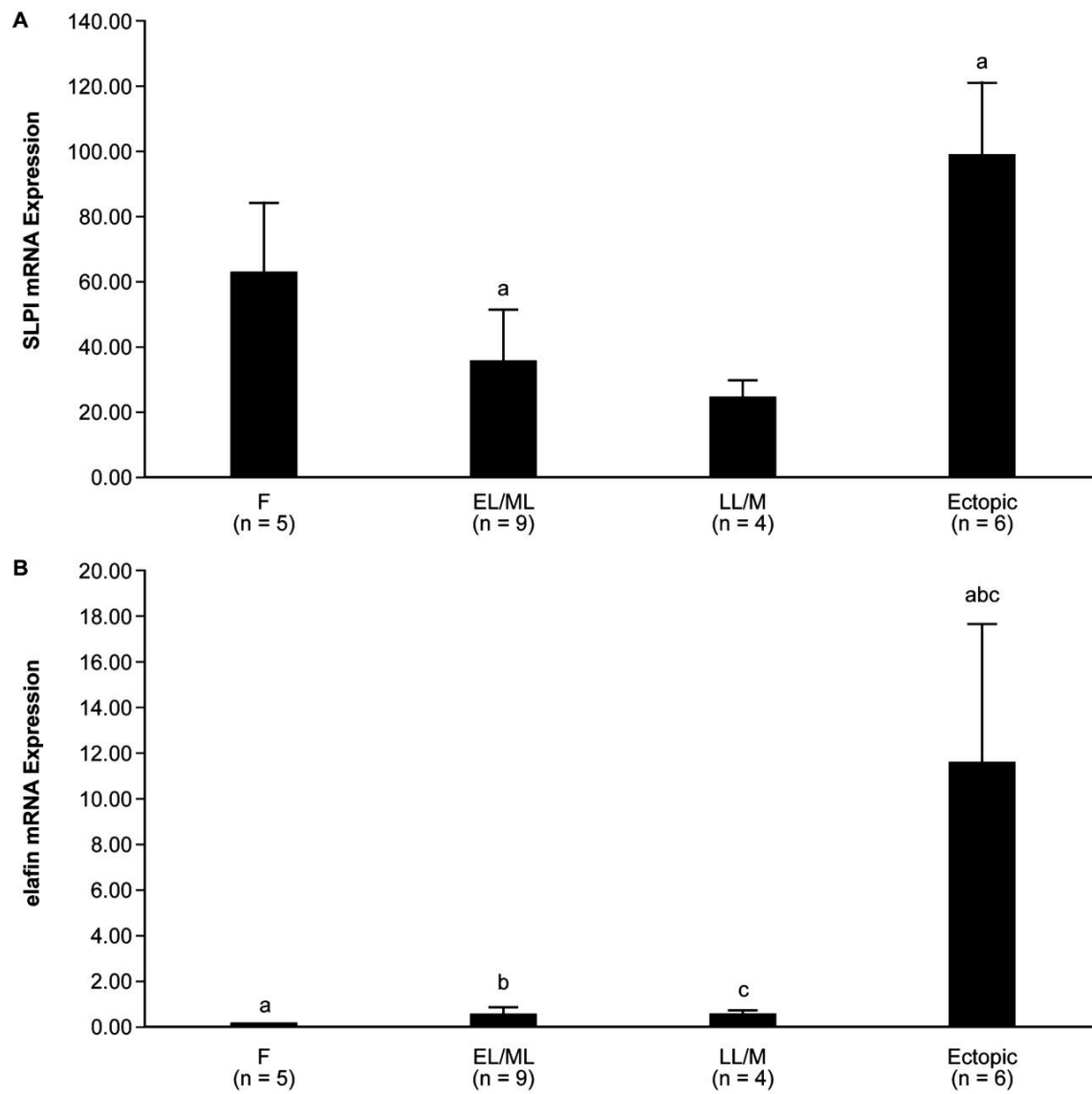


Fig.2

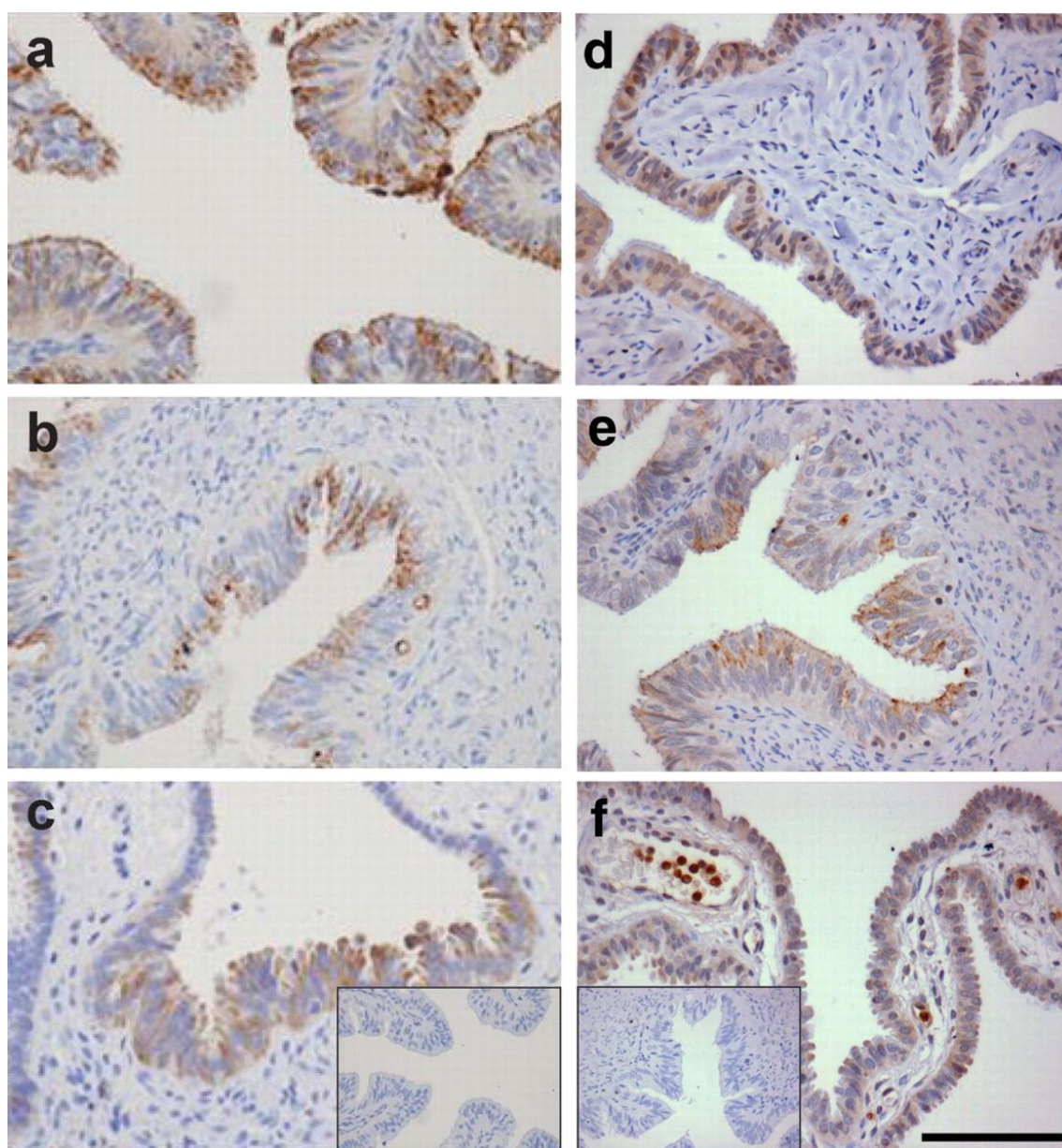


Fig.3

